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Approaching 'kit-type' labelling with ⁶⁸Ga: ~~The~~ the DATA chelators

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Abstract

The DATA chelators, are a novel class of tri-anionic chelators ligands based on ~~the~~ 6-amino-1,4-diazepine-triacetic acid, ~~core structure, that~~ have been introduced recently for the chelation of ⁶⁸Ga. Compared to ~~established~~ macrocyclic chelators based on the cyclen scaffold (i.e. DOTA-, DO3A- and DO2A- derivatives), they enable undergo quantitative radiolabelling both more rapidly and under ~~considerably~~ milder conditions. ~~To date, a detailed examination of the radiochemical characteristics of these chelators had not been carried out.~~ In this study, ~~we present~~ a systematic evaluation of the labelling of four DATA chelators – DATA^M, DATA^P, DATA^{Ph} and DATA^{PPh} ~~by~~ ⁶⁸Ga is presented. The results ~~of this study underline highlight~~ the extraordinary potential of this novel new class of chelators for application in molecular imaging using ⁶⁸Ga-~~PET~~ positron emission tomography (PET).

Introduction

Many researchers using positron emission tomography (PET) in the field have described the ⁶⁸Ga radionuclide as one with great potential and promise for widespread application in non-invasive clinical diagnosis of disease and infection ~~using positron emission tomography (PET)~~. (Antunes et al., Eur. J. Nucl. Med. Mol. Imaging, 2007, 34, 982-993; Al-Nahhas et al., Anticancer Research, 2007, 27, 4087-94; Al-Nahhas et al., Eur. J. Nucl. Med. Mol. Imaging, 2007, 34, 1897-1901; Oeberg, Endocrine, 2012, 42, 3-4; Al-Nahhas, EISO, 2009, 35, 561-67; Roesch F., Applied Radiation and Isotopes, 2013, 76, 24-30) The convenient half-life of 68 min, which is well suited to a variety of biochemical processes of interest, and attractive decay characteristics (90 % β⁺ yield, 1.9 MeV) make it a propitious alternative to established radionuclides such as ¹⁸F (PET) or ^{99m}Tc (SPECT). In more general terms, the superior spatial resolution of PET over SPECT provides greater sensitivity and image contrast. (Garcia, J. Nucl. Cardiol., 2012, 19, S19-29) The convenience, reliability and economy of the ⁶⁸Ge/⁶⁸Ga generator have specific benefits for locations where cyclotrons are not available, providing further impetus for the development of ⁶⁸Ga-radiopharmaceuticals. (Fani et al., Contrast Media Mol. Imaging, 2008, 3, 53-63)

Early research with ⁶⁸Ga dates back to the 1960s, but significant interest in this promising radionuclide ~~only corresponds with~~ was marked by the arrival of the modern ⁶⁸Ge/⁶⁸Ga generator in the 2000s. (Razbash A. A., Proceedings of the 5th International Conference on Isotopes, 2005, p. 147) Numerous obstacles to the clinical application of ⁶⁸Ga, such as ⁶⁸Ge breakthrough and metal impurities have been overcome in recent times, most notably the development of a GMP generator (Eckert & Ziegler, Berlin; iThemba, South Africa) and post processing methods suitable for clinical application (Eppard 2014 J. Nucl. Med; Zhernosekov, 2007, J. Nucl. Med., Mueller, 2012, Bioconjugate Chem.).

The recent promotion of ⁶⁸Ga-DOTA-TOC to orphan drug status in certain EU countries is evidence for the potential importance of ⁶⁸Ga-PET. (J. Nucl. Med., 2014, 55, 10N) In spite of this, and many other successes there is strong 'resistance' from more established radionuclides. ~~DOTA-based The~~

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⁶⁸Ga radiopharmaceuticals based on the octadentate ligand DOTA (1,4,7,10-tetraazacyclododecane-tetra-acetate) are regarded as the current 'industry standard', and fulfil ~~the certain~~ basic requirements: ~~that~~ incorporation of the radionuclide takes place within 15 min and gives rise to a complex of sufficient ~~in vivo~~ stability to resist premature metal dissociation in vivo. (Kubicek et al., Inorg. Chem., 2010, 49, 10960-10969). A disadvantage of DOTA based precursors is the relatively harsh conditions (100 °C, 15 min, pH < 4) required for ~~incorporation of the radionuclide~~ radiolabelling. This is due to a mis-match between the cavity size of the macrocycle, which is better suited to larger metal ions (i.e. ~~lanthanides~~ yttrium, the lanthanide ions and calcium), and ~~the small~~ ionic radius of Ga(III) (0.62 Å in 6-coordination vs 0.65 Å for high spin octahedral Fe³⁺). There is a belief ~~in the field~~ that in order for the ⁶⁸Ga radionuclide to achieve widespread application, and in the process ~~dislodge-displace~~ current standards, the radiolabelling protocol should align better with the favourable properties of the generator. Specifically, there is a drive towards the development of chelators which allow the preparation of 'ready-for-injection' ⁶⁸Ga-radiopharmaceuticals in a kit-type protocol. In this context, a kit is defined as a preparation in which the precursor and other components (bulking agents, buffer and radiolysis protectants) required for labelling are lyophilised in a vial. Labelling is initiated by the addition of the radioactive eluate which may be diluted with water as necessary. The ~~important boundaries~~ essential boundary conditions for use of the kit are set by the conditions used for labelling: ~~Quantitative-quantitative~~ labelling should occur in less than 10 min at room temperature, ~~a~~ precursor concentration and pH suitable for *in vivo* application should be used such that the injection is possible following sterile filtration – with the only external 'assistance' being a simple agitation.

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In order to ~~achieve this~~ meet these exacting conditions, the chelator should ~~enable-allow~~ quantitative incorporation of the radionuclide at room temperature both rapidly (< 10 min), and at a concentration and pH which are suitable for ~~further-wider~~ application. Such a labelling protocol has been described for ^{99m}Tc radiopharmaceuticals, and played a decisive role in ~~this-the establishment of this~~ radionuclide ~~becoming-as~~ the work-horse of nuclear medicine. (Kempi V. and Persson R. B., Nuclear-Medizin, 1975, 13(4), 389-399) A further advantage that stems from such a labelling protocol is that it would permit the preparation of ~~radiolabels-radiolabelled conjugates~~ previously inaccessible due to the temperature and/or pH sensitivity of the vectors involved. 'Kit'-type preparations which require elevated temperatures have been described (Mukherjee et al., J. Radioanal. Nucl. Chem., 2014, doi: 10.1007/s10967-014-3643-7); however the usefulness of such a high temperature labelling protocol is ~~brought into question~~ very limited with the commercially available ~~ility of~~ ⁶⁸Ga labelling modules.

~~In line with this aim~~ Accordingly, numerous-several research groups have ~~developed-sought new~~ chelator systems (e.g. TRAP, NOPO, HBED, NODAGA and H₂DEDPA) ~~which-that~~ show ~~remarkable promising~~ ⁶⁸Ga-labelling characteristics.

(FIGURE XX- ADD FIGURE HERE FOR EACH OF THESE STRUCTURES

) TRAP: Notni et al., Chem. Eur. J., 2011, 17, 14718-14722; NOPO: Simecek et al., Mol. Pharmaceutics, 2014, 11(11), 3893-3903; H₂DEDPA: Boros E. et al., JACS, 2010, 132, 15726-33; HBED: L'Eplattenier et al., JACS, 1967, 89, 837-843) As ~~unconjugated-the unsubstituted~~ chelators, all each one permits rapid and quantitative radiolabelling under mild conditions, ~~and-Indeed~~, in many cases the authors have alluded to the attractive prospect and potential for kit-type labelling. However, to the best of our knowledge, a kit-type labelling protocol for ~~a-each of these~~ ⁶⁸Ga-radiopharmaceuticals has not been reported. To some degree this is likely to be related to the profound effect that the targeting vector conjugation has on labelling efficiency, ~~which necessitates~~ the use of elevated temperature and/or a higher chelator concentrations.

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In 2012, Parker and co-workers introduced a series of four ~~hexadentate novel~~ chelators based on the 6-amino-1,4-diazepine triacetate (DATA) ~~core~~ (Figure 1), ~~that form octahedral complexes with Ga(III).~~ (Waldron, B. P. et al., Chem. Comm., 2013, 49, 579; Parker D. and Waldron B. P., Org. Biomol. Chem., 2013, 11, 2827; Waldron, B. P., Yufit, D S., Parker, D., Dalton trans., 2013, 42, 8001) The authors reported quantitative preparation of the ^{68}Ga complexes over the pH range 4 – 7 at room temperature (10 μM , 100 MBq ^{68}Ga) for ~~all four each~~ chelators, which are stable in the presence of apo-transferrin. Recently, Seemann and co-workers have shown that the favourable labelling characterises of DATA^M are retained when the ^{68}Ga generator eluate is processed by the acetone, ethanol or NaCl methods. (Seemann J. et al., Applied Radiat. Isot., 2015, Vol. 98, April, 54-59) The efficient and robust labelling ~~properties~~ makes them promising candidates for application in ^{68}Ga -PET and ~~in~~ the development of kit-type labelling.

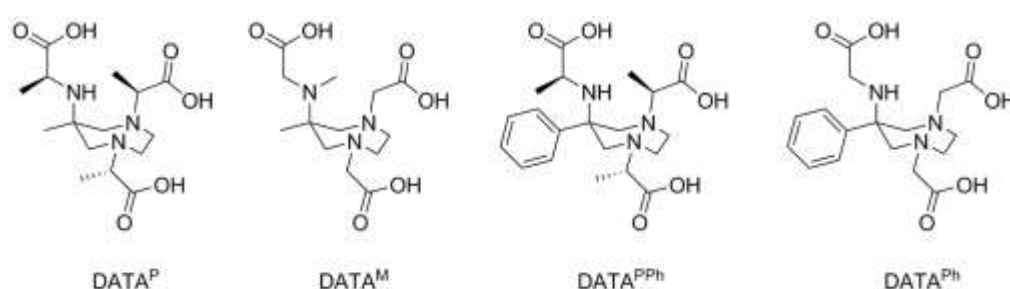


Figure 1 The four DATA chelators featuring the 6-amino-1,4-diazepine triamine scaffold—: **P** refers to propionate, and **M** and **Ph** to the methyl or phenyl substituent at the 6-position.

The initial communication provided a brief insight into the potential utility of the DATA chelators, but did not probe the limits of the labelling kinetics or identify the optimum conditions. (Waldron, B. P. et al., Chem. Comm., 2013, 49, 579) The original four DATA chelators feature subtle structural variations which were made primarily to alter relative lipophilicity and provide a better understanding of the optimum chelator identity through variation of the preferred ligand conformation and its degree of pre-organisation. The influence of the chirality and ~~chelator~~ lipophilicity of a chelator on the bio-distribution of a targeting vector is appreciated but poorly understood, and has not been investigated to any great extent. (Fani. M. et al., J. Nucl. Med., 2011 52 (7), 1110; Guerra Gomez, F. L. et al., Bioconjugate chem., 2012, 23 (11), 2229) Parker et al demonstrated that the radiolabelling characteristics and complex stability are not noticeably perturbed by these structural changes, which offers the interesting potential for chelators to be 'tailored' to the needs of the targeting vector and biological process. (Waldron, B. P. et al., Chem. Comm., 2013, 49, 579) However, moving towards the development of novel DATA-based bifunctional chelators it is important to ~~determine the optimal~~ identify the best chelating unit.

In this ~~report-work~~, we present an in-depth radiochemical evaluation and optimisation of the four DATA chelators. Specifically, this involves a comparison of their performance under ~~their-specified~~ limiting-labelling conditions—, as well as stability studies, competitive labelling in the presence of NOTA, metal ion selectivity experiments and ~~Log P~~ the determinations of log P values. In line with the labelling characteristics of the DATA chelators, and the development of kit-type protocol, ~~all~~ each evaluations of labelling ~~have-has~~ been carried out at ambient temperature.

Experimental

General

The four DATA chelators used herein were synthesised according to literature procedures. (Waldron, B. P. et al., Chem. Comm., 2013, 49, 579). DOTA and NOTA were purchased from ABX. All other reagents were purchased from commercial sources (Sigma-Aldrich® or Merck®) and used without further purification.

Radiochemical

All radiochemical evaluations were conducted using chemicals ~~in~~^{of} the highest available purity grade. Labelling and evaluations were performed at ambient temperature (unless otherwise stated), which was controlled to 22 – 23 °C by means of an air-conditioning unit.

A $^{68}\text{Ge}/^{68}\text{Ga}$ generator (TiO₂-based matrix, Cyclotron Co. Obninsk, Russia) was used for all experiments. The generator eluate was purified and pre-concentrated using a cation exchange-based post-processing. Post-processing was carried out according to a literature procedure using a washing solution (N1: 80 % acetone, 0.15 M HCl, 1000 µL) and eluting solution (N2: 97.56 % acetone, 0.05 M HCl, 400 µL). (Zhernosekov, 2007, J. Nucl. Med., Vol. 48, No 10, 1741) Briefly, the protocol involved elution of the generator with 0.1 M HCl (5 mL). The eluate was passed over a cation-exchange resin to trap ^{68}Ga whilst metal cation impurities contained in the eluate are not. Residual metal impurities were eluted from the resin with solution N1. The purified ^{68}Ga was eluted with solution N2 and used for radiolabelling without further modification.

Radiolabelling reactions were monitored using radio-TLC at time points of 1, 3, 5, 10 and 15 min. Radio-TLC plates (silica 60 F254, 4.5×4.5 cm, Merck) were spotted with 1 µL samples and developed using a 0.1 M citrate buffer (pH 4) as the mobile phase. Analysis was performed using a flat-bed imaging scanner (Instant Imager, Canberra Packard).

Measurements of pH were conducted using a calibrated pH meter (Mettler-Toledo, SevenEasy pH, Switzerland). All buffer solutions were tested to ensure the desired pH is obtained upon addition of the acidic eluate, and the final labelling pH recorded for each experiment. All experiments were performed in triplicate.

All radiolabelling experiments were conducted at 23 °C on a shaker-heating block (DITABIS MHR 11) at 400 rpm. The radiolabelling kinetics for each chelator was evaluated at different labelling pH and precursor concentration.

Concentration-dependent kinetics

10, 7 and 5 nmol of each chelator were dissolved in 1 mL of NaOAc buffer (0.2 M, pH 5) and placed on a shaker device, and the post-processed ^{68}Ga (400 µL, ~ 100 MBq) added.

pH-dependent kinetics

~~Solutions~~^{Buffer solutions of} at pH 4 (0.2 M NaOAc), 5 (0.2 M NaOAc), 6 (1.0 M NH₄OAc) and 7 (1.0 M NaHEPES) were prepared. In each case the aforementioned solution (1 mL) was added to ~~the chelator at a concentration of~~ 5 (DATA^M and DATA^P), 7 (DATA^{Ph} and DATA^{PPH}) or 15 (all) nmol ~~of chelator~~, and the post-processed ^{68}Ga (400 µL, ~ 100 MBq) added.

Stability studies (apo-transferrin, Fe(III), DTPA)

Stability experiments with the radiolabelled chelators were performed against DTPA, Fe(III) and apo-transferrin. Three separate challenge solutions (pH 7, PBS buffered saline) were prepared containing 5000 eq DTPA, 5000 eq Fe and 130 eq apo-transferrin with respect to the amount of DATA^x used for labelling. ~~An aliquot of~~ 200 µL of the challenge solution was preheated to 37 °C for 10 min on a heater-shaker device, and 50 µL of the radiolabelled chelator added. The radiolabelled complexes

were prepared at pH 4, 5, 6 and 7 using 12 nmol DATA^x at 23 °C. TLC samples (1 µL) were taken at 15, 30, 60, 90 and 120 min intervals.

NOTA challenge

Another study was performed to evaluate the preference of ⁶⁸Ga towards NOTA or DATA. The ligand NOTA (28 nmol) and a DATA^x ligand (28 nmol) were added to 1 mL of 0.2 M NaOAc buffer at pH 4. The solution was agitated with 400 µL N2 solution containing ⁶⁸Ga at room temperature and the ratio of ⁶⁸Ga-NOTA to ⁶⁸Ga-DATA^x was examined after 1, 3, 5, 10 and 120 min via radio-TLC.

Metal ion challenge (Fe(III), Cu(II), Ca(II))

The ⁶⁸Ga eluate was spiked with various metal ions to determine the critical concentration at which labelling yields greater than 95 % are not possible within in-10 min. Solutions of CaCl₂, FeCl₃ and CuCl₂ were prepared as 0.2 M solutions in 0.2 M NaOAc buffer. 14 nmol of DATA^x (14 nmol) was dissolved in NaOAc buffer (pH 4.5; 1 mL 0.2 M) NaOAc buffer (pH 4.5) and increasing/decreasing amounts of impurities, starting with 1 eq w.r.t that amount of DATA^x, added. The solution was incubated with 400 µL N2 solution (400 µL) containing ⁶⁸Ga for 10 min and radio-TLCs-TLC analysis was were used to evaluate the RCY-radiochemical yield at 10 min. The equivalents (eq) of the metal ion present during labelling were increased/decreased from 1 eq as follows. Ca(II): 2.0 eq; Fe(III): 0.5 eq; Cu(II): 0.1 eq.

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Lipophilicity studies

The lipophilicities of the four DATA chelators, DOTA and NOTA were determined using two different methods.

(i) Shake flask method

The shake-flask method is a ~~an octanol-water method~~ modified octanol-water method, adapted to suit relatively short-lived radionuclides.

After preparation of the radiolabelled chelator solution, the acetone content of the labelling solution was removed by evaporation at 95 °C. An aliquot of 20 µL of the resulting solution was added to a mixture of PBS (480 µL) and *n*-octanol (500 µL) in an Eppendorf vial. The mixture was agitated vigorously for 3 min at 900 rpm on a shaker device and centrifuged for 2 min at 600 rpm. Equal volumes (150 µL) of the PBS and *n*-octanol phases were extracted and 1 µL of each spotted on a TLC plate. Measurement of counts-per-minute (cpm) was performed using a Canberra Instant Imager. The lipophilicity (Log-log_P) is defined as the quotient of the activity in the organic and aqueous phases.

(ii) HPLC method

The HPLC method provides a qualitative comparison of the relative lipophilicities based on their column retention times. The method used was a slightly modified version of that described by Du and co-workers for the analysis of non-radioactive compounds. (C. M. Du, K. Valko, C. Bevan, D. Reynolds and M.H.Abraham, *Anal. Chem.*, 1998, 70, 4228-4234) Analytical HPLC conditions (LiChrospher 100-RP-18EC, 1 mL/min) used in the for lipophilicity studies ~~was-were~~ based on a gradient using water (A) and acetonitrile (B), each containing 0.1 % TFA. Mobile phase gradient: 0 – 1.5 min 1 % B, 1.5 – 10.5 min 99 % B, 11.5 min 99 % B, 12 - 15 min 1 % B. The HPLC instrument was coupled to a UV (Hitachi L-7400) and a radioactivity (Gamma Raytest) detection system. HPLC grade solvents were used and degassed by ultra-sonication for 15–20 min prior to use.

⁶⁸Ga-DOTA and ⁶⁸Ga-NOTA used for-in these this lipophilicity studies were prepared according to literature procedures. (I. Velykian, H. Maecke and B. Langstrom, *Bioconjugate Chem.*, 2008, 19(2), 569-573)

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Results

Concentration dependent kinetics:

Prior to starting this evaluation, a set of preliminary labellings were conducted to gain some insight into the optimum pH for radiolabelling. These suggested that most efficient chelation occurred at pH 5, and therefore this was used as the labelling pH in this for the evaluation.

The labelling kinetic time dependence of the radiolabelling yield (pH 5, 1 mL 0.2 M NaOAc) for each chelator at concentrations 10, 7 and 5 nmol are shown in (figures-Figures 2 to 4) respectively. There is an revealed the expected decrease in the rate of radiolabelling for each chelator as the amount used decreases with reduction in concentration, as well as a greater degree of standard deviation. Radiochemical yields in excess of 98 % over the course of 10 minutes, and greater than 94 % after only 1 minute, were achieved with for 7 and 10 nmol chelator concentrations of the chelators. There was a decrease in the rate with 5 nmol of the chelator, and that was most pronounced for DATA^{Ph} and DATA^{PPh}. Although labelling was slightly slower, overall yields for DATA^M and DATA^P were above 93 and 97 % after 1 and 10 minutes. DATA^{Ph} and DATA^{PPh} achieved similar yields of ~ 84 and ~ 93 % at 1 and 10 minutes respectively. Most efficient labelling was displayed by DATA^M which labelled to > 97 % in 1 minute with 5 nmol chelator.

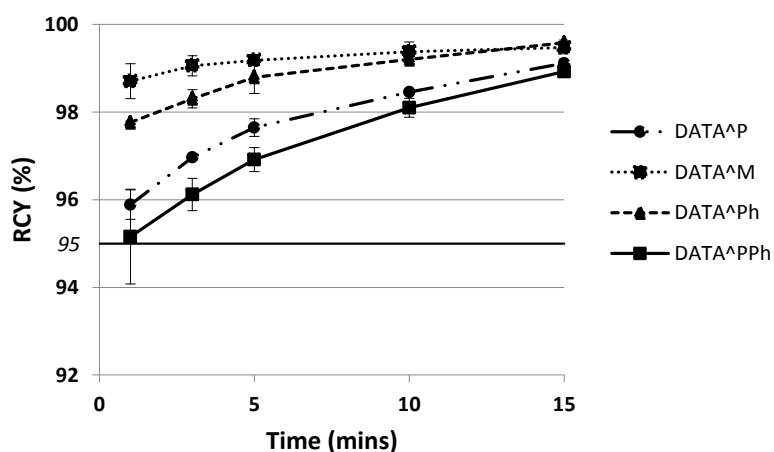


Figure 2 Radiolabelling Time dependence of radiolabelling with ⁶⁸Ga using 10 nmol chelator (pH 5, 23 °C).

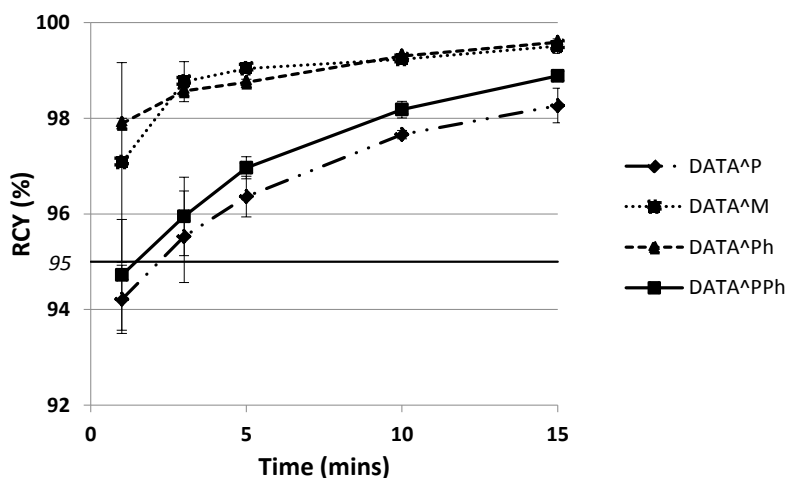


Figure 3 Radiolabelling Time dependence of radiolabelling with ^{68}Ga using 7 nmol chelator (pH 5, 23 °C).

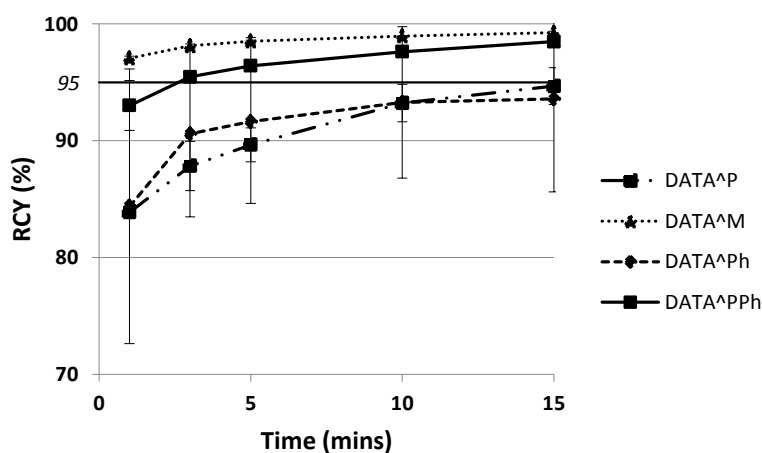


Figure 4 Radiolabelling Time dependence of adiolabelling with ^{68}Ga using 5 nmol chelator (pH 5, 23 °C).

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pH dependent kinetics:

Initially the influence of pH was evaluated using a 15 nmol concentration of the chelator; conditions which replicated our earlier work. (Waldron, B. P. et al., Chem. Comm., 2013, 49, 579) Under these conditions all four each chelators achieved > 97 % RCY after only 5 min. However, this does such conditions do not provide information regarding the optimum pH for radiolabelling. To gain further insight, the evaluation was carried out using lower chelator concentrations at which the rate of radiolabelling is being limited but still able to achieve RCYs > 95 %.

Accordingly, the radiolabelling of the triacetate chelators (DATA^{Ph} and DATA^{M}) was performed using at 5 nmol, and 7 nmol for the tripropionic-triisopropionate bearing chelators (DATA^{P} and DATA^{PPH}) 7 nmol. The labelling kinetics obtained at pH 4, 5, 6 and 7 are shown in figures-Figures 6 to 9 respectively. For an internal comparison of each chelator the RCY at 5 and 15 min is shown at each pH in f(Figure 10). In general terms, optimum labelling is achieved at pH 5, which gave rise to RCYs >93 and 97 % after 1 and 15 min respectively for all chelators. With the exception of DATA^{Ph} there was a noticeable decrease in the rate of reaction-complexation at pH 4, however-However, RCYs over 5 min remained high at > 98 %. The rate decreased more significantly at pH 6 resulting in noticeably reduced RCYs over 15 min with the exception of DATA^{P} ; achieving a yield of 93 % after 15 min. All-Each chelators showed improved kinetics at pH 7 (compared to pH 6), but only DATA^{P} achieved an acceptable yield (98 %, 15 min) with the other chelators in the range 80 – 87 % (15 min). Figures 6 – 9 show how the kinetics of the different chelators vary at each pH. Figure 10 shows the RCY at 5 and 15 min for each DATA chelator across the pH range 4 – 7. Cross-chelator comparisons can only be made for the pairings DATA^{M} - DATA^{Ph} and DATA^{P} - DATA^{PPH} , since the amount of chelator used is otherwise was not the same.

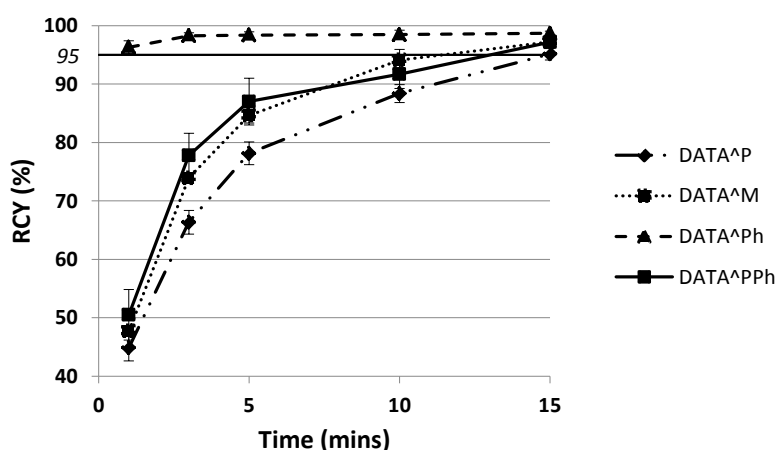


Figure 6 Radiolabelling Time dependence of radiolabelling at pH 4 (23 °C) for DATA^{M} (7 nmol), DATA^{P} (7 nmol), DATA^{Ph} (5 nmol) and DATA^{PPH} (7 nmol).

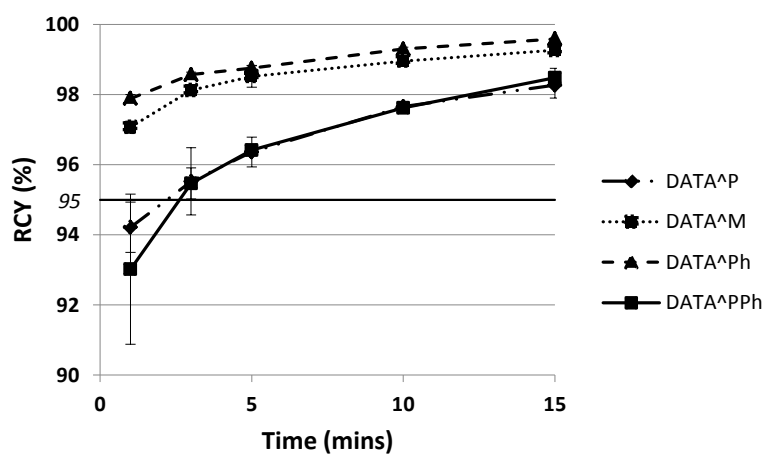


Figure 7 ~~Radiolabelling Time dependence of radiolabelling kinetics~~ at pH 5 (23 °C) for DATA^M (7 ~~nmol~~), DATA^P (7 nmol), DATA^{Ph} (5 nmol) and DATA^{PPh} (7 nmol).

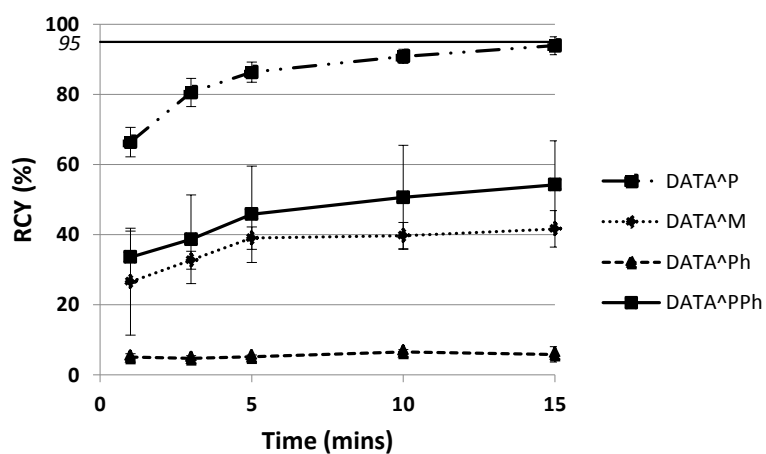


Figure 8 Radiolabelling Time dependence of radiolabelling at pH 6 (23 °C) for DATA^M (7 nmol), DATA^P (7 _____ nmol), DATA^{Ph} (5 nmol) and DATA^{PPH} (7 nmol).

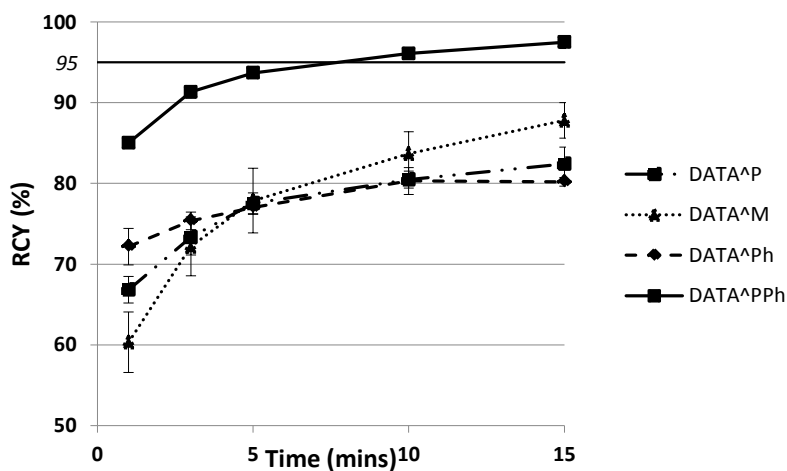


Figure 9 radiolabelling Time dependence of radiolabelling at pH 7 (23 °C) for DATA^M (7 nmol), DATA^P (7 nmol), DATA^{Ph} (5 nmol) and DATA^{PPh} (7 nmol).

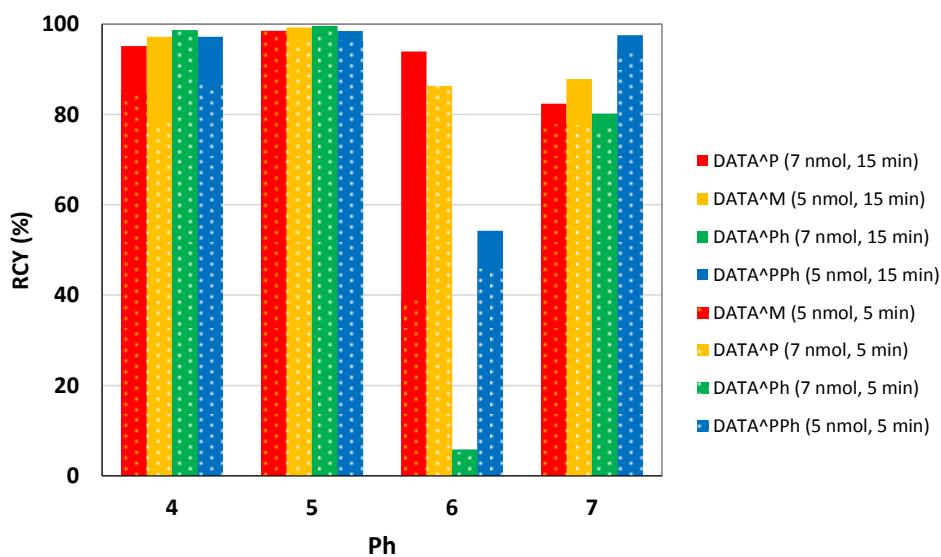


Figure 10 Variation of RCYs at pH 4, 5, 6 and 7 for DATA^M (5 nmol), DATA^P (7 nmol), DATA^{Ph} (5 nmol) and DATA^{PPh} (7 nmol) after 5 and 15 min radiolabelling at 23 °C.

Stability:

Radiolabelled chelators were prepared at pH 4, 5, 6 and 7 using 14 nmol of the chelator. In each case the stability was assessed separately for trans-chelation (*apo*-transferrin and DTPA) and trans-metalation (iron(III)) under physiological temperature and pH (37 °C and pH 7) conditions in phosphate buffered saline. In all cases every case, there was < 3% instability (i.e. activity not

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associated with the non-radiolabel that remained within the range of the background signal intensity).

NOTA competition:

The objective of this evaluation was to assess the relative rates of radiolabelling ~~of for~~ NOTA and DATA^x, as well as the susceptibility to trans-chelation after radiolabelling has taken place. ~~This-The latter issue was achieved-addressed~~ by adding the activity to a cocktail containing NOTA and DATA^x in equimolar amounts. The optimal labelling conditions for NOTA were used for this experiment (12 nmol of each chelator, pH 4.5, ambient temperature) even though previous experiments showed that these were not the best for the DATA^x chelators. The DATA^M, DATA^P and DATA^{PPh} ligands each exhibit show comparable labelling kinetics to ~~that of~~ NOTA, chelating 65, 56 and 53 % of the ⁶⁸Ga present respectively. However, ⁶⁸Ga-DATA^{PPh} displayed a ~~somewhat~~ faster rate of labelling than NOTA, by chelating 76 % of the available ⁶⁸Ga. The only other active spot visible in these analyses was due to ⁶⁸Ga-NOTA. The relative population of the different radiolabels did not change ~~of over~~ the course of 120 min, indicating that ~~the respective each~~ ⁶⁸Ga complexes ~~were was~~ stable with respect to trans-chelation, over this time ~~framescale~~.

Metal ion challenge:

~~Table 2 shows the results obtained for the metal ion competition studies.~~ The objective of ~~this these~~ experiments was to assess the influence of metal-ion impurities on the rate of radiolabelling, and therefore provide some insight into the preference of the chelators for ⁶⁸Ga. The results obtained for the metal ion competition studies are summarised in Table 2. The 'equivalents' refer to the DATA^x chelator, and therefore represent a significant excess, relative to the amount of ⁶⁸Ga used. The data shown represents the maximum amount of metal ion which can be present and still achieve > 95 % radiolabelling. The chelators showed similar level of tolerances to the ~~presence oif the~~ different metal ions examined. Copper(II) showed the greatest influence, whilst calcium(II) had virtually no effect on the rate of radiolabelling. Labelling was possible in the presence of iron(III), but reached a critical level when > 3.5 – 4 eq w.r.t DATA^x was present. The values given (Table 1) shown in the table are the maximum equivalents of the metal ion at which RCYs of > 95 % ~~after 10 min are were~~ achieved after 10 minutes.

Table 1 Equivalents of different metal cations which can be present without reducing RCY at 10 min to < 95 %.

Chelator	Metal ion present (eq w.r.t DATA ^x)		
	Ca(II) ¹	Fe(III)	Cu(II)
DATA ^P	8 x 10 ⁶	4	1.2
DATA ^M	8 x 10 ⁶	3.5	1.25
DATA ^{PPh}	8 x 10 ⁶	4	0.75
DATA ^{PPh}	8 x 10 ⁶	4	0.75

¹ Higher amounts of calcium were not tested as the values exceeded what would be experienced *in vivo* and during labelling.

Lipophilicity:

The Log P values determined using the shake-flask method are shown in Table 2, ~~in order of increasing lipophilicity from top to bottom.~~

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Table 2 log P values determined using the modified octanol-water shake-flask method.

Chelator	Log P
DATA ^M	- 4.02 ± 0.16
DATA ^P	- 2.91 ± 0.05
DATA ^{Ph}	- 2.68 ± 0.17
DATA ^{PPh}	- 2.30 ± 0.08

The results of the HPLC lipophilicity studies ~~carried out using HPLC~~ are also shown below, for purposes of comparison, shown graphically in figure(Figure 11). The peaks heights are arbitrary and have been normalised ~~for the purpose to allow of a direct~~ comparison. Under the conditions used, it is expected that the more hydrophilic ⁶⁸Ga-complexes are eluted earlier.

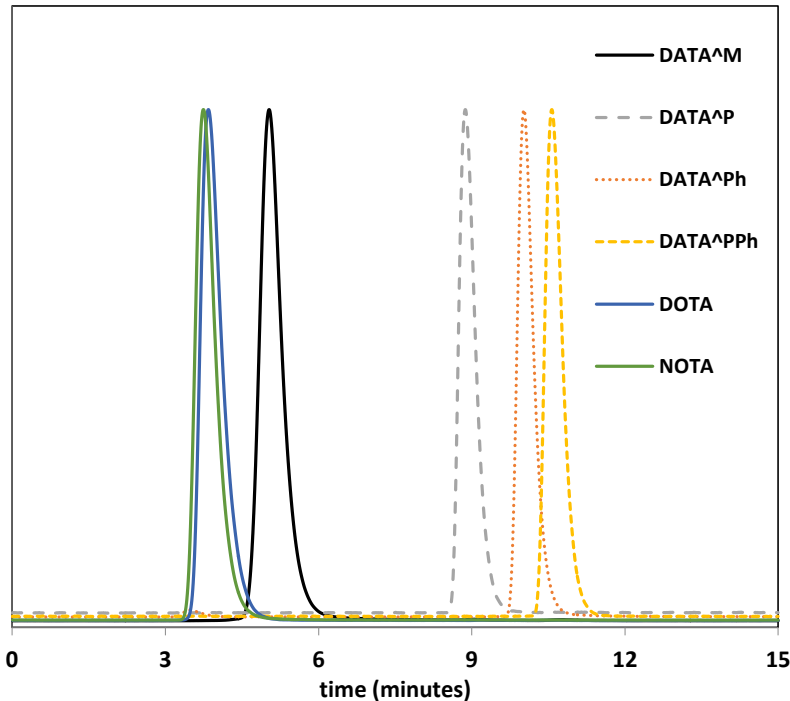


Figure 11 Retention-HPLC retention times of ⁶⁸Ga-DOTA chelators, in comparison with ⁶⁸Ga-NOTA and -DOTA.

Discussion

Metallo-radiopharmaceuticals typically consist of the chelator, radionuclide and targeting vector. The chelator is responsible for stabilising the radionuclide and ~~providing-provides~~ a means for labelling the targeting vector. Conjugation of the radiolabelled complex to a targeting vector provides a bio-distribution profile of interest. These targeting vectors are often relatively large biomolecules which direct the radionuclide to regions/processes of interest, and can have an untoward effect on the radiolabelling performance of the chelator. These negative side effects can be overcome by using a higher labelling temperature and/or the use of a higher concentration of

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~~more~~-precursor. Therefore, to maintain efficient room temperature labelling it is important to determine the optimum conditions for each chelator.

The development of a kit-type labelling protocol has been one of our primary goals, and as a result ~~all each~~ radiolabelling evaluations ~~have been~~was carried out at ambient temperatures ~~without external heating~~.

In radiopharmacy, especially with ~~the~~ shorter-lived radionuclides, time is a critical factor. In order to preserve as much as possible of a radionuclide's 'life' ~~as possible~~ and minimize the radiation dose to the patient, a radiochemical preparation should be as short-quick as possible. Purification strategies for radiopharmaceuticals are time consuming and can be avoided if the RCY high-is sufficiently enough. Within radiochemical circles, a yield of > 95 % is considered to be sufficient, such that subsequent purification is not necessary, and is defined as being an acceptable yield.

Each of the DATA^x chelators displays remarkable radiolabelling characteristics with > 95 % RCYs possible over the pH range 4 – 7 within 3 min using 15 nmol (10.7 μ M) of the chelator at room temperature. The current 'industry standard', DOTA, requires 10 – 15 min at 95 °C to achieve similar RCYs. This combination of a superior rate of reaction and room temperature labelling is particularly attractive in the view of a kit-type approach to radiolabelling. The ability to radiolabel over a wide pH range is attractive as it provides both a more robust labelling reaction, ~~the radiolabel~~ at a pH suitable for in vivo administration (time-saving) and may also enable the synthesis of previously inaccessible pH sensitive biomolecules. The DATA chelators are characterised by a triamine scaffold which is a hybrid of acyclic and cyclic components. ~~It is believed~~Conformational analysis studies using variable temperature NMR had suggested that that the acyclic portion imparts ~~a certain degree of sufficient~~ flexibility ~~which to~~ lowers the energy barrier to metal ion complexation, whilst the cyclic character of the diazepine ring pre-organises the chelator ~~in to adopt~~ a conformation ~~for that aids~~ faster complex formation. The degree of pre-organisaiton is higher for the ligands with a more sterically bulky 6-phenyl substituent.

The dependence of the radiolabelling yield on chelator concentration was determined for each chelator at pH 5. Reducing the amount of chelator from 10 (7.1 μ M) to 7 nmol (5.0 μ M) had a negligible influence on the rate of reaction and final RCY for each chelator. The RCYs for each chelator after 1 min ~~where~~ > 94.5 % and there ~~is was~~ less than 2.5 % variation between ~~the each~~ chelators after 5 min. The decrease in labelling efficiency is more noticeable when the amount of chelator ~~is was~~ reduced to 5 nmol (3.6 μ M), with DATA^{ph} and DATA^p falling to 93 % after 10 min. DATA^M and DATA^{pph} retained the remarkable labelling characteristics even at 3.6 μ M, with yields of 97 (1 min) and 95 % (3 min) respectively.

At 7 and 10 nmol levels, the ~~propionic acid~~propionate bearing chelators displayed lower labelling rates than the acetate ~~bearing chelators~~analogues. This trend ~~falls away~~was less marked when 5 nmol of chelator ~~is was~~ used ~~where and~~ the labelling efficiency of DATA^{ph} and DATA^p drops-off to a greater extent. However, caution should be exercised when considering the results ~~because for the following reasons.~~ (1) Standard deviations become more significant at low concentrations, and (2) even at a 5 nmol concentration, there ~~is is~~ typically less than 10 % separating the RCY after 3 min. Looking at the kinetic profiles ~~of for~~ DATA^M and DATA^{ph} (Figures 6 to 9) it is evident that the a significant portion of the final RCY ~~is was~~ achieved within the first minute. In contrast, the labelling profile of chelators bearing the propionic acid pendant arms ~~increases~~increased more steadily over time, suggesting that the rate of reaction is slightly slower. Presumably, this ~~is may be~~ a result consequence of the reduced conformational mobility of the propionic acid arms arising from steric interactions ~~of with~~ the α -methyl groups ~~with of~~ the diazepine ring and adjacent pendant groups, ~~which reduces~~leading to a reduction in the rate ~~at which of~~ complexation ~~takes place~~. It is interesting

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to note that the labelling profile of DATA^M and DATA^{Pph} does not change as significantly with decreasing chelator concentration. In contrast, there is a more discernible change for DATA^P and DATA^{Ph} when the chelator is reduced to 5 nmol. ~~Graphs-Additional kinetic profiles,~~ comparing the labelling kinetics for each chelator at 5, 7 and 10 nmol are ~~supplied-given~~ in the SI.

Due to the rapid rates of radiolabelling, it was necessary to carry out the pH-dependent kinetic studies using a chelator concentration which limited the rate of reaction. Therefore, the lowest chelator amount which permitted 95 % labelling after 10 min was used. For DATA^M and DATA^{Pph} this was 5 nmol, and 7 nmol for DATA^P and DATA^{Pph}. ~~There is a~~The common optimum pH ~~value~~ for all DATA^X chelators ~~of-is~~ pH 5. ~~At this pH -which facilitated~~ yields of > 95 % ~~were obtained~~ after only 3 min for DATA^P and DATA^{Pph}, and 1 min for DATA^M and DATA^{Ph}. The rate of the labelling at pH 4 ~~decreases-decreased~~ significantly ~~such that RCYs at 1 min are < 50 %~~, with the exception of DATA^{Ph} which ~~achieves-gave rise to~~ a RCY 95 % after 1 min, ~~such that RCYs at 1 min are < 50 %~~.

In spite of the decreased rate of reaction, acceptable yields ~~are-were~~ still achieved for all chelators (< 95 %) after 15 min. At pH 6, there was a significant decrease in efficiency, such that none of the chelators achieved the critical yield of 95 % after 15 min. The DATA^P ligand performed significantly better than the other chelators with a yield of 93 % after 15 min. Curiously, the radiolabelling efficiency increased at pH 7 for DATA^M, DATA^{Ph} and DATA^{Pph}, but only DATA^{Pph} achieved a > 95 % yield after 15 min. Figure 10 shows how ~~the-The variation of RCYs-RCY with pH~~ for each chelator ~~vary-with~~ pH, (Figure 10) ~~and~~ suggests that chelators based on the methyl substituted scaffold are ~~general~~ more resilient to pH changes than those ~~of-based on~~ the phenyl substituted scaffold. There is no obvious reason why the labelling efficiency decreases at pH 6, but it is possible that it may be related to nature of the buffer used. At pH 4 and 5 sodium acetate is used for the buffer solution, but at pH 6 ammonium acetate ~~is-was~~ used to achieve the desired labelling pH. Acetate does not function effectively as a buffer at pH 6 and there is also the ammonium cation present in solution. It is possible that these features played a role in the reduced labelling yields. There is scope to improve the yields by increasing the amount of chelator used, most notably for DATA^P and DATA^M which may be a tolerable trade-off, given the benefits of being able to radiolabel at higher pH.

The 9N₃ scaffold of NOTA provides the ideal framework for the formation of ~~a-and~~ distorted octahedral coordination cage preferred by the ~~small~~ Ga(III) cation, and as a result, provides highly efficient labelling and exceptionally high kinetic stability. The ligand NOTA ~~is-has been regarded as~~ the standard for ~~room temperature~~ labelling of ⁶⁸Ga for some time, and has been successfully translated to NODAGA-TOC. (Eisenwiener, K.-P. et al., Bioconjugate Chem., 2002, 13, 530-541) In a direct competition experiment, the labelling efficiency of the DATA chelators compared favourably with NOTA, ~~by-chelating-a~~ greater portion of the available ⁶⁸Ga ~~was chelated in a direct competition experiment~~. Furthermore, there was no evidence for transchelation of ⁶⁸Ga from any of the radiolabelled complexes over 2 hours.

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Competition ~~for the chelator~~ between ⁶⁸Ga and other ~~trace~~ metal ions ~~for the chelator~~ can diminish the labelling yield, and is particularly relevant ~~issue in~~ radiopharmacy where low concentrations of the radionuclide are used. A considerable amount of work has been done to determine the metal ion contamination in the ⁶⁸Ga used for labelling, as well as ways in which these can be removed. Interestingly, there are comparably fewer reports in which the influence of the metal ions is investigated in detail. Notni and co-workers investigated the influence of zinc(II), copper(II), iron(III), aluminium(III), tin(IV) and titanium(IV) on the labelling on a set of macrocyclic ~~phosphinate~~ chelators. In general they found that iron(III) and copper(II) had the most significant effect on the labelling. (Simecek, J. et al., Chem. Med. Chem., 2013, Jan 7, 8(1), 95-103) Ca(II) is not considered a metal ion of concern during radiolabelling, but is present *in vivo* in relatively high concentrations (2 mM) and ~~it was~~ therefore of interest to understand its ~~predisposition for the chelator effect~~. In ~~our test~~ this work, the presence of 8.0 M calcium(II) had no detectable influence on labelling.

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Iron(III) is present in ^{68}Ga eluates at concentrations of up to 50 nM (Simecek. J. et al., Chem. Med. Chem., 2013, Jan 7, 8(1), 95-103) and is of concern due to its chemical ~~similarities~~ similarity to the gallium(III) ion. Radiolabelling in the presence of iron(III) was affected to a greater extent, but this was only evident when > 3.5 – 4 equivalents of the metal ion (w.r.t DATA^x) was used. ~~copper~~ Copper(II) was also tested due to its exceptionally high affinity for tri-basic nitrogen chelator (*viz.* the Irving-Williams series), and provides the most stringent test of the chelators. DATA^p and DATA^M could tolerate 1.2 and 1.25 equivalents of copper(II) respectively, whilst ~~DATA^{ppH}~~ DATA^{ppH} and DATA^{ph} showed diminished radiolabelling with 0.75 equivalents. Taking into account the large excess of the metal ion contaminants (at least μM) in relation to the amount of ^{68}Ga present (1 nM) the chelators have displayed a relatively high affinity for gallium over the other metal ions tested.

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The lipophilicities of the radiolabelled complexes displayed ed the expected trend based on their structural characteristics, and the trend was consistent between the two independent methods used. In order of increasing lipophilicity, they are the observed order was: DATA^M, DATA^p, DATA^{ph} and DATA^{ppH}. The range of lipophilicities offered by the four chelators is quite remarkable with HPLC retention times ranging from 5 to 10.5 min. Therefore, an additional benefit of these DATA chelators is that the chelator can be tailored to the needs of the application.

Summary and Conclusion~~conclusions~~

The optimum DATA chelator has been identified as DATA^M ~~which it~~ can be radiolabelled in 1 minute at ambient temperature using 5 nmol of the chelator. The other DATA chelators performed only marginally ~~worse~~ less well, requiring 7 nmol and 5 min to achieve a similar feat. There is also scope to use the pH range 4 - 7 by increasing the amount of chelator used ~~and or~~ using slightly longer reaction times, whilst remaining within the confines of what is acceptable in terms of time (15 min) and chelator concentration (11 μM).

~~A~~ The wider labelling range at ambient temperature offers a more robust labelling protocol than existing alternatives, but also augurs well for the ability to label conjugated biomolecules which are temperature and/or pH sensitive. The radiolabelled complexes are stable to trans-chelation (NOTA, DTPA and apo-transferrin) and transmetallation (iron(III)). There is also considerable variation in the lipophilicity of the four ~~chelators~~ chelator complexes, ~~which could provide valuable insight into the importance of the physical properties of the chelator, that may have implications inn their individual biodistributions in vivo.~~

~~The~~ In summary, the DATA chelators display radiolabelling characteristics which make them ideal candidates for the development of a new generation of ^{68}Ga -PET imaging agents which can be labelled in a kit-type manner. Current work is focused on the development of a conjugated bifunctional derivative based on DATA^M ~~; and has shown~~ very encouraging results ~~thus have been obtained for that will reported subsequently.~~

Acknowledgements We thank the Association of Commonwelath Universities for a scholarship (BPW), xxx

References

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Supplementary information

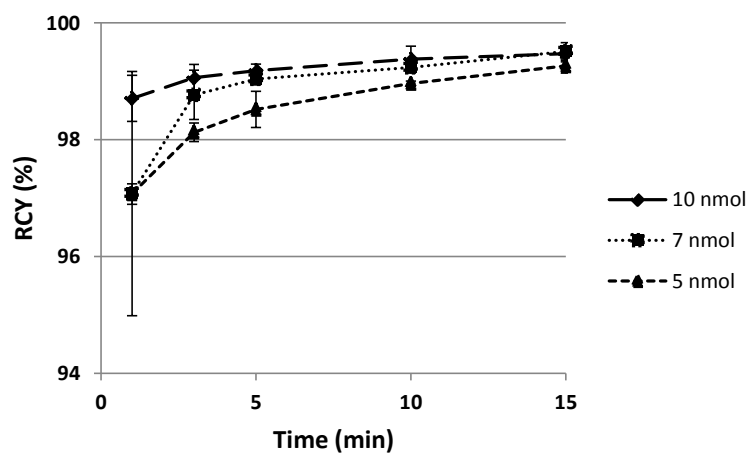


Figure S1 Time dependence of radiolabelling of for DATA^M at 23 °C and pH 5 with 10, 7 and 5 nmol of chelator.

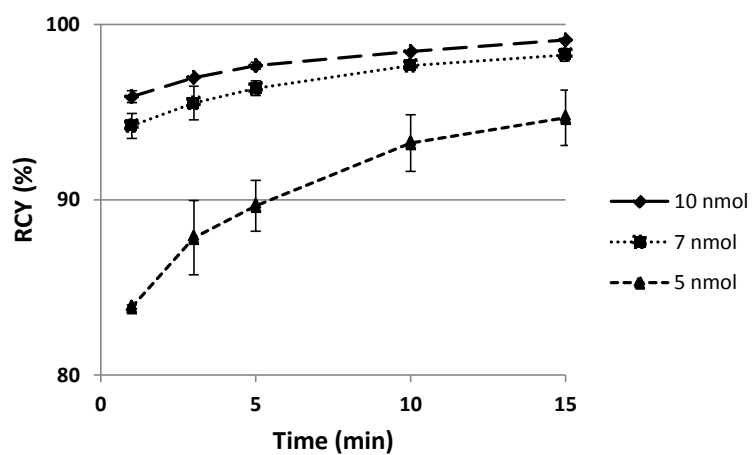


Figure S2 ~~Radiolabelling~~ Time dependence of radiolabelling of for DATA^P at (23 °C and, pH 5 with, 10, 7 and 5 nmol)., ~~chelator~~.

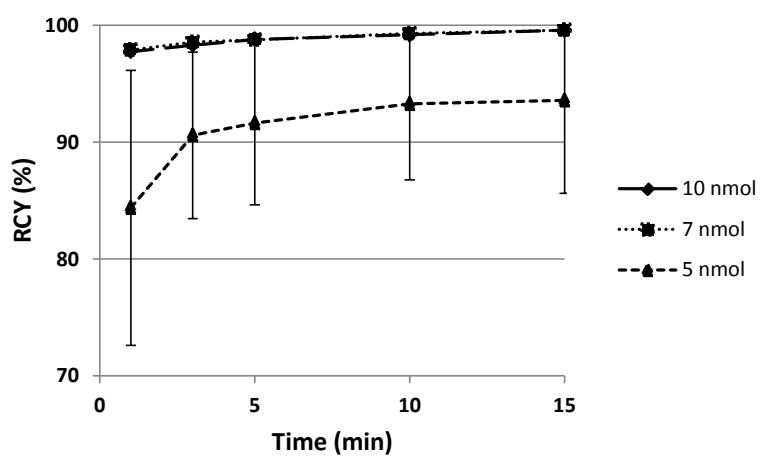


Figure S3 ~~Radiolabelling~~ Time dependence of radiolabelling of for DATA^{Ph} at (23 °C and, pH 5 with, 10, 7 and 5 nmol)., ~~chelator~~.

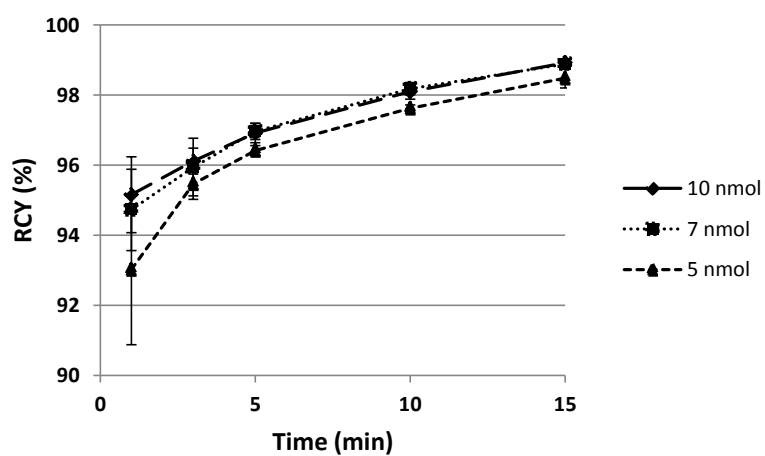


Figure S4 Radiolabelling Time dependence of radiolabelling of for DATA^{pH} at (23 °C and, pH 5) with 10, 7 and 5 nmol chelator.

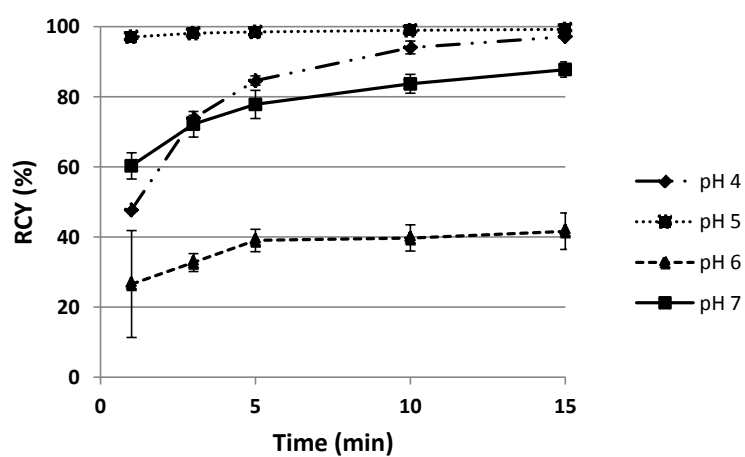


Figure S5 Radiolabelling of DATA^M (5 nmol) at 23 °C and pH 4, 5, 6 and 7.

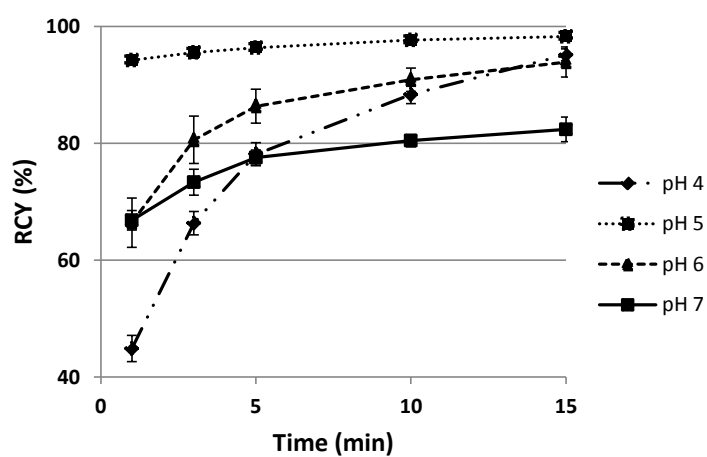


Figure S6 Radiolabelling of DATA^P (7 nmol) at 23 °C and pH 4, 5, 6 and 7.

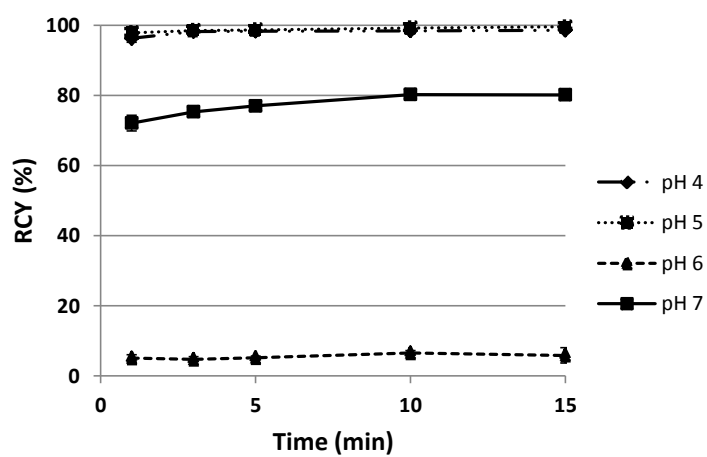


Figure S7 Radiolabelling of DATA^{Ph} (7 nmol) at 23 °C and pH 4, 5, 6 and 7.

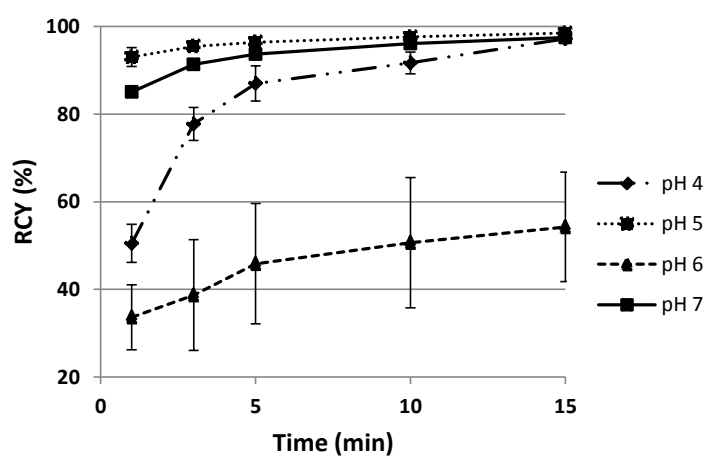


Figure S8 Radiolabelling of DATA^{pph} (5 nmol) at 23 °C and pH 4, 5, 6 and 7.